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(54) Title: PROCESS OF PRODUCTION OF A PREPARATION FOR STAINING NUCLEAR DNA, PREPARATION OBTAINED THEREBY AND KIT FOR ITS USE

(57) Abstract

A process for the production of a preparation for specific staining of nuclear DNA is disclosed, that can be used to determine the DNA cell contents by flow cytometry. The working solution of such a preparation, containing besides the specific dye, a thermolabile enzyme and a surfactant, is dehydrated under vacuum so as to stabilize it and to modify its characteristic of access to ligand and hydration, and in this way it can be stored for a long time, preferably packed in single dose vials, and then reconstituted at the moment of use, maintaining its properties perfectly unaltered.

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"PROCESS OF PRODUCTION OF A PREPARATION FOR STAINING NUCLEAR DNA, PREPARATION OBTAINED THEREBY AND KIT FOR ITS USE"

The present invention relates to a process for the production of a preparation for staining nuclear DNA to be used in flow cytometry, the preparation obtained by such a process, and a kit for its use.

It is well known that cytofluorometric analysis took a fundamental role in diagnostics and its use is steadily increasing, together with the development of new and more sophisticated analysis techniques and apparatus.

More particularly, the determination of cellular DNA contents through flow cytometry is fundamental in the oncologic field as well as in determining the cellular ploidy by cytofluorometric analysis.

Up to now the working solution containing the specific dye and a thermolabile enzyme, was prepared in an extemporaneous way at the time of using it, because of the well known stability problems of the proteins in the anhydrous condition, but such a process is not at all satisfactory because it is also well known that extemporaneous preparations depend too much from the present situation and the operator's skill, thus they do not warrant reproducible results.

Indeed, as proteins in the anhydrous condition generally show stability problems and their rehydration is rather long, difficult and sometimes incomplete, it was hitherto considered impossible to make a preparation for staining nuclear DNA to be stored in the anhydrous condition and to be reconstructed at the time of use.

It was now surprisingly found that this problem is brilliantly solved by mixing the protein with a surfactant, so as to cause the formation of a water envelope around the molecule, obtain a better stability with time and a more complete

hydration at the moment of use.

In other words, not a true lyophilization but instead a crystallization of the preparation in carried out, so that it may be perfectly stored for a long time.

The ingredients to be used in the production of the preparation according to the present invention must be chosen obviously in a proper way. As enzyme a specific ribonuclease is used, for instance type A bovine ribonuclease; as a dye, a fluorescent halide binding specifically to nucleic acids, preferably propidium iodide, but other fluorescent halides are suitable as well; as a surfactant, it is necessary to use a non ionic surfactant such as NONIDET P 40 (BDH Laboratory Supplies Ltd., Poole, Dorset, Great Britain).

Once the working solution is prepared, it is delivered in vials in such amount as to allow an analytic determination for each vial, which is therefore single dose, and the solution water is evaporated under vacuum.

It is known that DNA staining may take place either in a hypotonic medium, if it is desired to stain only isolated nuclei, or in an isotonic medium, if it desired to keep intact the cell structure for possible morphologic studies or checking proliferation markers.

Two different diluents are therefore prepared for reconstituting the working solution, consisting of salts with a different concentration according to either diluent.

Consequently, three different single dose kits may be supplied to users, namely the vial containing the preparation, accompanied by a bottle containing either the isotonic or the hypotonic diluent, or two vials each with one of the diluents.

The production of the preparation according to the present invention will be

now illustrated in detail in the following practical example of preparation, which however should not be construed as limiting in any way the scope of the invention.

EXAMPLE

The chosen dye is propidium iodide, that links specifically by intercalation to the nucleic acids in their double strand structure; it is therefore necessary to remove the cell RNA by enzymatic digestion, using a specific ribonuclease, so as to avoid an overestimation (moreover the RNA contents of a cell is not constant, but it may vary with the state of activity).

Access of the dye to the nuclear compartment is made easier by using a surfactant, present at a low concentration, which makes the nuclear membrane permeable.

Firstly a high concentration stock solution of propidium iodide is prepared, dissolving 500 μg/ml of the dye in twice distilled water. The mixture so obtained is left standing on a magnetic agitator for at least 4-6 hours so as to obtain the complete dissolution of the dye. The solution is then filtered with a membrane of cellulose acetate with a pore size 0.22 μ so as to remove possible insoluble residues. Subsequently the working solution is prepared: to one part of stock solution, 9 parts of twice distilled water are added (the final concentration of the dye results to be 50 μg/ml), 1 mg/ml of crystallized bovine ribonuclease type A and 0.1% v/v NONIDET P 40. The mixture is again placed on a magnetic agitator for at least 2 hours, adjusting the magnet speed so as not to create foam, and then filtered with a membrane of cellulose acetate with a pore size 0.22 μ. The working solution is delivered in conic bottom test tubes having a capacity of 1.5 ml, in a quantity of 1 ml for each tube. Water evaporation is obtained using a thickener

with a fixed angle rotor, with a low speed centrifugation (1500 rpm) for 8 hours at controlled temperature (25°C) under vacuum. At the end the test tubes are closed with a proper pressure stopper.

Two different diluents are then prepared for the reconstitution of the working solution. Isotonic diluent No. 1: 0.155 M NaCl, 15 mM NaN_3 in twice distilled water. Hypotonic diluent No. 2: 0.01 M NaCl, 15 mM NaN_3 in twice distilled water. Both solution, after complete dissolution on magnetic agitator, are filtered with a membrane of cellulose acetate with a pore size of 0.22 μ for removing possible insoluble residues and sterilization from bacterial contaminants.

CLAIMS

1) Process of production of a preparation for staining nuclear DNA, constituted by a working solution containing a specific dye and a thermolabile enzyme for RNA digestion, characterized by the steps of mixing said enzyme with a surfactant and evaporating water of the working solution under vacuum, so as to obtain a crystallization residue that may be stored for a long time and reconstituted at the moment of use by suitable diluents.

2) Process according to Claim 1, characterized in that a fluorescent halide is used as specific dye.

3) Process according to Claim 2, characterized in that propidium iodide is used as fluorescent halide.

4) Process according to Claim 1, characterized in that a non ionic surfactant is used as a surfactant.

5) Process according to Claim 4, characterized in that the product marketed under the name NONIDET P40 is used as non ionic surfactant.

6) Process according Claim 1, characterized in that a specific ribonuclease, is used as enzyme.

7) Process according to Claim 6, characterized in that bovine ribonuclease type A is used as ribonuclease.

8) Process according to anyone of Claims 1 to 7, characterized in that a hypotonic or isotonic medium is used as a diluent for the reconstitution of the working solution at the moment of use.

9) Preparation for staining nuclear DNA, characterized in that it comprises the crystallization residue of a working solution containing a specific dye, a thermolabile enzyme for RNA digestion and an enzyme stabilizing surfactant, to

be reconstituted at the moment of use by means of a hypotonic or isotonic medium.

10) Preparation for staining nuclear DNA, whenever obtain through the process according to one or more of Claims 1 to 8.

11) Preparation for staining nuclear DNA according to Claim 9 or 10, characterized in that it is packed in single dose vials allowing only one analytic determination for each vial.

12) Kit for using the preparation of Claim 11, characterized in that the single dose vial is accompanied by a bottle containing either the isotonic or the hypotonic diluent, or by two bottles each containing one of said two diluents.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N1/30 C12Q1/68 C12N9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF HISTOCHEMISTRY, vol. 37, 1993 page 68 BARBESTI ET AL. 'dna flow cytometry performed by stabilised propidium iodide staining' see the whole document ---	1-7
A	US,A,4 087 328 (SWAISGOOD) 2 May 1978	1
A	see column 7, line 64 - line 66 see column 2, line 50 - column 3, line 12 --- -/--	8,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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Internat Application No
PCT/EP 94/03217

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	IMMUNOPHARMACOLOGY, vol. 28, no. 3, 18 October 1994 AMSTERDAM, pages 241-257, A.BROWN ET AL. 'propellant -driven aerosols of functional proteins' see page 242, column 1, line 46 - column 2, line 38 see page 253, column 2, line 4 - page 254, column 1, line 9	1
A	BIOCHIMICA ET BIOPHYSICA ACTA , vol. 947, 1988 pages 209-246, LUISI ET AL. 'reverse micelles as hosts for proteins and small molecules' see page 232, column 2, last paragraph - page 235, column 2, paragraph 3 see page 241, paragraph XI - page 242	1
A	US,A,4 668 618 (THORNTHWAITE) 26 May 1987 see column 2, line 2 - line 20 see column 4, line 7 - line 49 see column 6, line 39 - line 43	2-5

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Information on patent family members

Internat ' Application No
PCT/EP 94/03217

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4087328	02-05-78	NONE	
US-A-4668618	26-05-87	US-A- 4906561	06-03-90